

Opposing RA and FGF signals control proximodistal vertebrate limb development through regulation of *Meis* genes

Nadia Mercader¹, Esther Leonardo¹, María Elisa Piedra², Carlos Martínez-A¹, María Ángeles Ros² and Miguel Torres^{1,*}

¹Departamento de Inmunología y Oncología, Centro Nacional de Biotecnología, CSIC-UAM, E-28049 Madrid, Spain

²Departamento de Anatomía y Biología Celular, Universidad de Cantabria 39011 Santander, Spain

*Author for correspondence (e-mail: mtorres@cnb.uam.es)

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SUMMARY

Vertebrate limbs develop in a temporal proximodistal sequence, with proximal regions specified and generated earlier than distal ones. Whereas considerable information is available on the mechanisms promoting limb growth, those involved in determining the proximodistal identity of limb parts remain largely unknown. We show here that retinoic acid (RA) is an upstream activator of the proximal determinant genes *Meis1* and *Meis2*. RA promotes proximalization of limb cells and endogenous RA signaling is required to maintain the proximal *Meis* domain in the limb. RA synthesis and signaling range, which initially span the entire lateral plate mesoderm, become restricted to

proximal limb domains by the apical ectodermal ridge (AER) activity following limb initiation. We identify fibroblast growth factor (FGF) as the main molecule responsible for this AER activity and propose a model integrating the role of FGF in limb cell proliferation, with a specific function in promoting distalization through inhibition of RA production and signaling.

Key words: Limb, Proximodistal patterning, *Meis1*, *Meis2*, Retinoic acid, *Raldh2*, Fibroblast growth factor 8, *Hox*, Apical ectodermal ridge, Chick

INTRODUCTION

Vertebrate limbs provide an excellent model for the study of developmental mechanisms, since several molecules involved in its growth and patterning have been identified (Cohn and Tickle, 1996; Johnson and Tabin, 1997; Schwabe et al., 1998). However, little is known of how limb cells acquire their identity along the proximodistal axis. Limb growth takes place in a proximodistal (PD) sequence, such that elements closer to the trunk are specified and differentiate earlier than those located further from it (Saunders, 1948; Summerbell et al., 1973). The sequential generation of limb parts is coupled to proliferation during their specification period. Generation of limb cells during this phase is brought about by proliferation in the progress zone (PZ), a distal mesenchymal region located just beneath the apical ectodermal ridge (AER). With time, proliferating cells in the PZ contribute cells to progressively more distal regions along the PD limb axis. Positional identity is acquired in the PZ and once a cell exits this region, its PD identity is fixed (Summerbell et al., 1973). Progenitor cells in the PZ thus progress with time through all the different PD identities, from the most proximal to most distal. Proliferation and distalization of PZ cells is promoted by AER signals (Saunders, 1948), comprising several members of the FGF family (Cohn et al., 1995; Fallon et al., 1994; Niswander et al., 1993). Proliferation is thus coupled to PD identity specification, however, there is no evidence as to how specific

AER signals control the program for progressive PZ cell distalization.

Two closely related homeobox genes, *Meis1* and *Meis2* (Cecconi et al., 1997; Moskow et al., 1995; Nakamura et al., 1996; Oulad-Abdelghani et al., 1997), have recently been identified as determinants of proximal limb compartments (Capdevila et al., 1999; Mercader et al., 1999). In limb development, expression of these two genes is observed in the lateral plate mesoderm and later in the proximal limb region, up to the stylopod-zeugopod (S-Z) boundary. The related homeobox *Pbx1* (Kamps et al., 1990; Nourse et al., 1990), a molecular and functional partner of *Meis* proteins whose nuclear import requires *Meis* (Berthelsen et al., 1999; Capdevila et al., 1999; Mercader et al., 1999), shows similar proximal restriction of its nuclear localization domain in developing limbs (González-Crespo et al., 1998; Mercader et al., 1999). Overexpression of either *Meis1* or *Meis2* leads to inhibition or truncation of distal limb compartments (Capdevila et al., 1999; Mercader et al., 1999). In addition, ectopic distal *Meis1* expression inhibits the progressive distalization of PZ cells, resulting in limbs with proximally shifted identities along the PD axis (Mercader et al., 1999). Similarly to *Meis/Pbx1*, the activity of the respective *Drosophila* orthologs HTH/EXD (Rauskolb et al., 1993; Rieckhof et al., 1997) is restricted to proximal limb domains and their ectopic expression inhibits or proximalizes distal limb regions (Abu-Shaar and Mann, 1998; González-Crespo et al., 1998; González-Crespo and Morata,

1996; Mercader et al., 1999). Furthermore, EXD also requires HTH for nuclear import (Abu-Shaar et al., 1999; Rieckhof et al., 1997). These results suggest a notable similarity of the molecular mechanisms controlling proximodistal limb development in metazoans.

Since normal regulation of *Meis* activity along the PD limb axis is essential for the correct distalization of limb cells, the mechanisms involved must play a central role in PD limb specification. Although combinations of distal diffusible factors, such as bone morphogenetic protein (BMP) plus FGF, or BMP plus Wnt, have been shown to inhibit *Meis* expression and to distalize proximal limb cells (Capdevila et al., 1999), the mechanism that promotes *Meis* activity in the proximal limb remains unknown. Here we explored this mechanism and found that retinoic acid (RA), a form of the vitamin A, is an activator of *Meis* genes. RA can induce changes in pattern of the three main limb axes (reviewed in Tickle and Eichele, 1994). Along the PD limb axis, RA has been shown to proximalize regenerating urodele limbs, leading to tandem PD duplication of limb structures (Maden, 1982) and, in the chick, grafts of limb tissue exposed to RA develop structures of a more proximal identity than expected (Tamura et al., 1997). We show here that RA activates ectopically *Meis* genes in the distal limb, promotes proximalization of limb cells and is required endogenously to maintain the proximal *Meis* domain in the limb. We found that, after limb-bud emergence, RA synthesis and signaling is restricted to the proximal limb by FGF activity. We propose a model for proximodistal limb development, in which FGF activity promotes limb distalization through inhibition of the proximalizing RA signal required to maintain the *Meis* activity. Our results provide a molecular basis for the role of RA in specifying proximal limb fates in different vertebrates, and suggest a mechanism by which FGF promotes limb distalization.

MATERIALS AND METHODS

In situ hybridization and cartilage staining

White Leghorn chicken embryos were used for all experiments. Whole-mount in situ hybridization was performed as described (Wilkinson and Nieto, 1993), using the following probes: gene names and nucleotides used were *cMeis1*: +439 to +1491, *cMeis2*: +532 to +999, *cHoxa13*: +487 to +1159, *cHoxa11*: +616 to +1160, *cRarb*: +1 to +1593, *cRaldh2*: +487 to +1186, *mMeis1*: +1 to +1398. Riboprobes *cBmp2*, *cBmp4* and *cBmp7* were kindly provided by C. Tabin and B. Houston.

Alcian green cartilage staining was performed as described (Morgan et al., 1992).

Bead implantation

AG1-X2 ion exchange resin beads (150–200 µm diameter; Biorad) were soaked for 20 minutes in all-*trans*-retinoic acid (Sigma) dissolved in dimethyl sulphoxide (DMSO). Beads were stained in Neutral Red for 10 minutes and rinsed three times in saline solution. Subapical bead insertion was performed at a distance of 150 µm from the most distal border of the bead to the AER. Beads were inserted as previously described (Ros et al., 2000) at different developmental stages as detailed in the figures and text. For the migration assay, RA beads were briefly dipped in a drop of DiI dissolved in DMSO before implantation. The retinoid antagonist AGN 193109 (Allergan Sales, Irvine, CA) was used at a concentration of 2.5–10 mg/ml in DMSO. Control beads were incubated in DMSO alone and treated as

described above for RA. Heparin-coated acrylic beads (Sigma) were rinsed in phosphate-buffered saline and incubated (room temperature, 1 hour) with 0.5 mg/ml recombinant mouse FGF-8b (R&D Systems) or 0.1 mg/ml recombinant BMP2 (Genetics Institute, MA, USA) on a culture dish. For FGF signaling inhibition, AG1-X2 beads were soaked for 1 hour in 2 µl of the FGFR1 inhibitor U5402 (Mohammadi et al., 1997) diluted in DMSO.

In general, one bead was implanted at a midposition along the anteroposterior axis. In some cases, up to three beads were implanted at the same proximodistal level. This was performed to assure a uniform modification along the anteroposterior axis.

AER removal and tissue grafts

For AER removal, the distal tip of the limb bud was lightly stained with Nile blue and the AER was completely removed with a Tungsten needle as described (Ros et al., 2000).

For tissue grafts, donor embryos were injected with *RCAS-mMeis1b* (Mercader et al., 1999) or *RCAS-GFP* (Logan et al., 1998) at stage 8–10 as described (Logan and Tabin, 1998). Donor-infected embryos were dissected at stage 23–24. The distalmost part of limb buds was collected in PBS and AERs were removed with a micro-fine dissecting knife. To remove the ectoderm from mesodermal pieces, fragments were digested for 10 minutes in 0.5% trypsin and transferred to 10% FCS in PBS. The mesenchyme was cut into small pieces and lightly stained with Nile blue. Host embryos of a similar stage were windowed and a small hole was made in the vitelline membrane to expose the limb. A donor mesenchyme fragment was inserted into a subapical incision of the host bud. After 48 hours, embryos were either processed for in situ hybridization to detect viral *mMeis1* expression, or directly examined under epifluorescence to detect viral GFP expression.

Photography

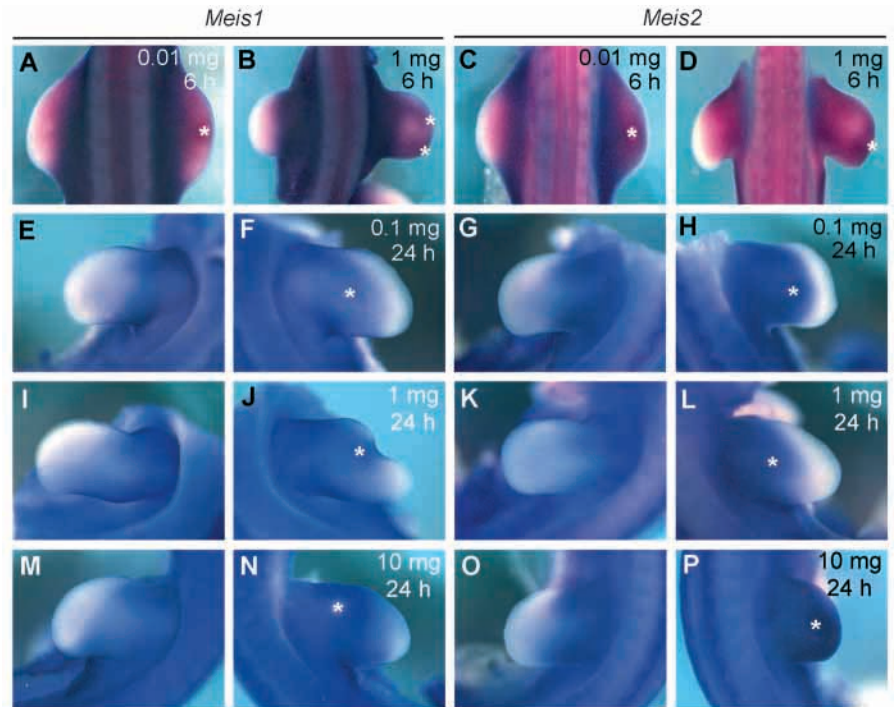
Whole-mount specimens were photographed under a binocular microscope with a “ProgRes 3008” digital camera (Kontron Elektronik). Fluorescent specimens were photographed under both epifluorescent and transmitted light in an inverted microscope with a “Colour CoolView” digital camera (Photonic Science).

RESULTS

Differential RA signaling along the PD axis of the developing limb determines the proximal restriction of *Meis* genes expression

Meis1 and *Meis2* genes are expressed along the lateral plate mesoderm before limb initiation. Shortly after limb-bud emergence, around Hamburger-Hamilton (HH) stage 19, an area with no *Meis* expression is seen at the distalmost end of the limb bud (Capdevila et al., 1999; Cecconi et al., 1997; Mercader et al., 1999; Oulad-Abdelghani et al., 1997). The *Meis*-negative area grows as the distal limb domains are generated with its limits fixed at the S-Z boundary (Mercader et al., 1999). Since *Meis2* responds to RA in vitro (Oulad-Abdelghani et al., 1997), we tested whether RA signaling was required for regulation of *Meis* expression in the limb by inserting RA-loaded beads in the distal regions of developing chick limbs. We found that, following implantation of beads soaked in 0.01 mg/ml RA, a concentration considered to represent a physiological level (Helms et al., 1994), both *Meis1* and *Meis2* messengers were upregulated in the distal limb (Fig. 1A,C). Different RA doses, in the 0.1 to 10 mg/ml range, induced *Meis* activation until at least 24 hours after treatment (Fig. 1E–H). Activation was dose-dependent (Fig. 1) and rapid

Fig. 1. Retinoic acid induces ectopic Meis expression in the distal limb bud. Whole-mount in situ hybridizations of control (E,G,I,K,M,O and left wing buds in A-D) and RA-treated limbs (F,H,J,L,N,P, and right wing buds in A-D) are shown. Limbs were hybridized with *cMeis1* (A,B,E,F,I,J,M,N) or *cMeis2* (C,D,G,H,K,L,O,P) riboprobes. Beads soaked in RA concentrations from 0.01 mg/ml (shown as 0.01 mg) to 10 mg/ml (shown as 10 mg) in DMSO were implanted in stage 19-21 chicken limbs. Implantations were performed subapically in all cases except in J in which the bead was placed immediately under the AER. Fixation was performed after 6 to 24 hours as indicated in each panel. Asterisks indicate bead location.



at any RA dose used, being evident from at least 4 to 6 hours after bead implantation (Fig. 1A-D; data not shown). Previous studies (Summerbell, 1983; Tickle et al., 1982, 1985) showed that application of RA beads beneath the anterior AER leads to duplication in the anteroposterior (AP) axis at moderate RA doses, and to limb truncations at higher RA concentrations, especially when applied distally. This phenotype correlated with Meis activation in the apical ectoderm (Fig. 1I,J). To avoid the negative effect upon the AER, we inserted the beads into the distal mesenchyme about 150 μ m apart from the AER, a position referred hereafter as subapical. When RA beads were applied subapically in the distal mesenchyme, limb growth was not abolished, RA-activated Meis-expressing cells incorporated to the limb PD axis, and a novel Meis-free domain was generated distally (Fig. 1F,N,H,L). With this procedure, we thus obtained local reprogramming of distal cells to express Meis genes through RA activation.

These results showed that RA can activate Meis genes ectopically in the distal limb region. To determine whether this activation is relevant in the maintenance of Meis expression in its endogenous proximal domain, we used a potent RA-specific antagonist (RAA) (Kochhar et al., 1998). Insertion of beads embedded in RAA (2.5-10 mg/ml), either into the proximal limb region or the lateral plate, led to repression of endogenous *Meis1* and *Meis2* mRNA expression in these regions (Fig. 2A-D). Repression was rapid, being already evident by 4 hours after bead implantation (Fig. 2E,F). We thus conclude that endogenous Meis expression is maintained by RA signaling, and that stronger RA signaling in proximal versus distal limb regions may determine the proximal restriction of Meis expression. In agreement with this view, the expression of *retinaldehyde dehydrogenase 2* (*Raldh2*), encoding the main RA-synthesizing enzyme, is restricted to the lateral plate mesoderm and most proximal region of the limb bud (Swindell et al., 1999 and see below Fig. 7E-G). In addition, *retinoic acid*

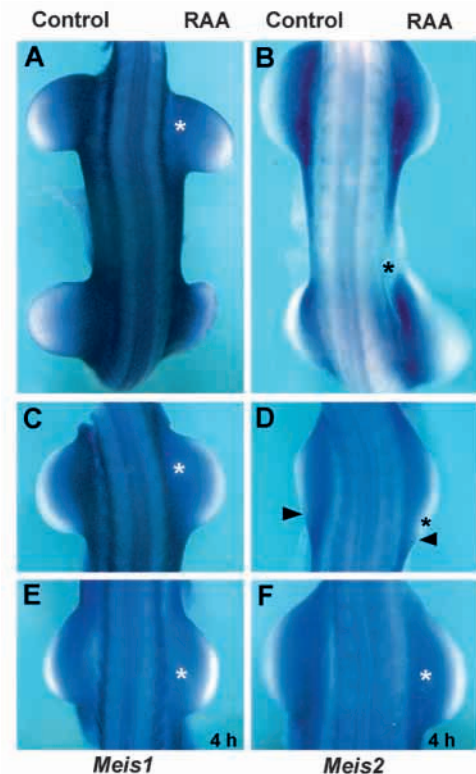


Fig. 2. Retinoic acid antagonist inhibits Meis expression within the proximal limb and lateral plate mesoderm. Beads loaded with 10 mg/ml RAA were implanted into the proximal mesenchyme of right wing buds of stage 20 embryos (A,C,E,F), or the right LPM of stage 15-17 embryos (B,D). Whole-mount in situ hybridization was performed 24 hours (A-D) and 4 hours (E,F) after operation, with *cMeis1* (A,C,E) or *cMeis2* (B,D,F). Asterisks indicate bead location. Arrowheads in D mark the posterior limit of the distal Meis-negative domain.

receptor β (*Rar\beta*), a previously identified in vitro and in vivo RA target gene (de The et al., 1990; Mendelsohn et al., 1992b; Rossant et al., 1991; Sucov et al., 1990), is restricted to the proximal limb in a domain comparable to that of Meis genes (Dollé et al., 1990; Mendelsohn et al., 1992a).

Retinoic acid reprograms the affinity of distal limb cells, promoting their incorporation into proximal compartments

Since *Meis1* overexpression proximalizes limb cells (Mercader et al., 1999), RA reprogramming of distal cells to express Meis genes should promote their proximalization. To determine whether this is the case, we analyzed the consequences of RA-induced local activation of Meis genes. RA beads were inserted subapically at sequential stages of limb development (HH 19 to 27) to achieve bead incorporation at different positions along the PD limb axis. Operations before stage 20 led to the incorporation of the bead proximal to the S-Z boundary, and generally produced no alterations in limb patterning (Fig. 3A). Remarkably, when RA beads were inserted between stages 22 and 24, they were incorporated to positions more proximal than those expected

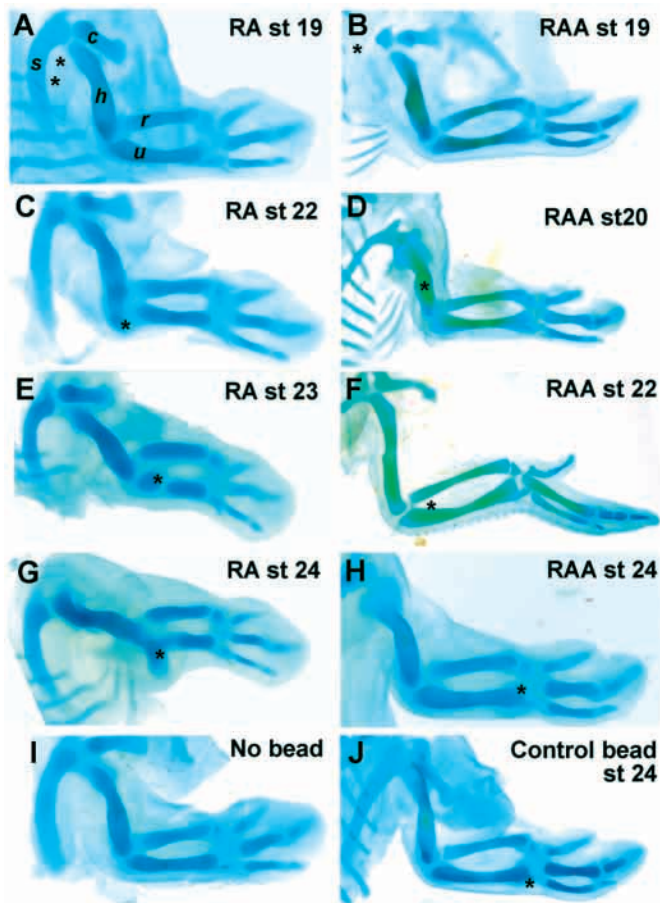


Fig. 3. Effect of retinoic acid and retinoic acid antagonist on bone patterning. RA (A,C,E,G), RAA (B,D,F,H) or control beads (J) were applied at different developmental stages and cartilage stainings were performed at stage 30–32. (I) Untreated stage 30 control limb. Asterisks indicate bead location. c, coracoid; h, humerus; r, radius; s, scapula; u, ulna.

for the time of operation. RA-free control beads inserted at stages 21–22 ended up at variable levels along the zeugopod, while they were found at the zeugopod-autopod (Z-A) boundary if inserted at stages 23–24 (Fig. 3J and data not shown). In contrast, RA beads inserted at stages 22–24 always incorporated close to the S-Z boundary (Fig. 3C,E,G).

Results in both amphibian and chicken models have shown that self-affinity is a property of limb cell PD identity, which determines their allocation to the corresponding PD level (Crawford and Stocum, 1988; Ide et al., 1994). Our results thus suggest that RA-exposed distal cells reprogrammed their PD affinity and moved proximally to reach the stylopod region. To test this possibility, we inserted control and RA beads that were dipped in DiI to label the cells surrounding the bead at the time of operation (stage 23). RA beads were inserted subapically and thus limb truncation was not observed in any case. RA beads were found to reach the S-Z boundary 48 hours after insertion (Fig. 4C,D; 16 out of 17 cases); in contrast, control beads remained at the Z-A boundary (9 out of 11 cases) or at the distal zeugopod (2 out of 11 cases) (Fig. 4A,B). In all cases, DiI-labelled cells were found in a single, discrete domain surrounding the RA bead (Fig. 4A–D), showing that limb cells exposed to RA contributed to more proximal structures than expected based on their original position. In contrast, operations after stage 25 led to RA bead incorporation to the autopod, with no obvious deviation from the expected position (not shown). RA can thus either only reprogram zeugopod cells, or the

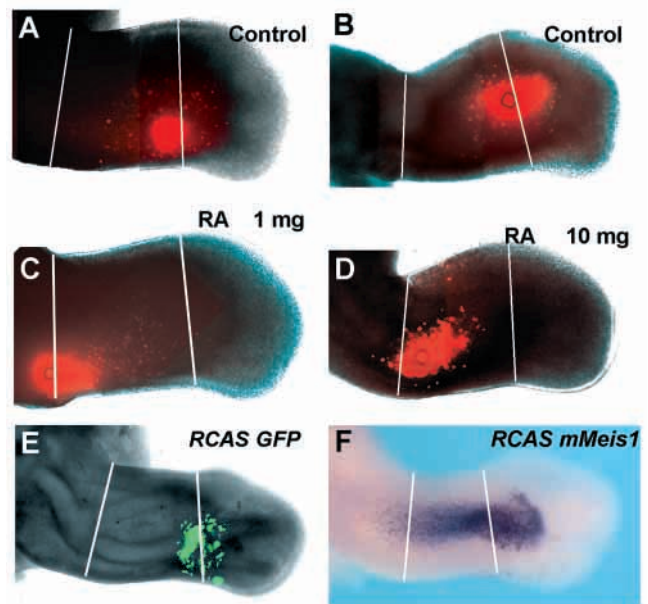


Fig. 4. Reprogramming of distal limb cell affinity by retinoic acid (RA). Control beads (A,B) or beads loaded with 1 mg/ml (C) or 10 mg/ml RA (D), were briefly dipped in DiI and implanted into the distal mesenchyme of stage 23 chicken limbs. Embryos were fixed after 48 hours and analyzed with a fluorescence microscope. (F) Isochronic and isotopic graft of *RCAS-mMeis1*-infected stage 23 distal mesenchyme cells. In situ hybridization to detect virus-derived *mMeis1* shows proximal relocation of Meis-expressing cells. (E) A similar experiment using a control *RCAS-GFP* virus shows that grafted cells remain at the wrist area. (A–E) Composites of bright-field and dark-field images. White lines indicate the S-Z and Z-A limits.

autopod may not provide an adequate environment for reprogrammed cells to relocate to more proximal compartments.

To test whether *Meis1* activation was responsible for proximal relocation of limb cells after RA exposure, we used *RCAS-mMeis1*-infected limbs as tissue donors in transplant experiments. We dissected distal mesenchyme from stage 23 infected limbs and performed isochronic and isotopic transplants to the subapical region of uninfected limbs. Detection of *RCAS*-derived *mMeis1* showed that, after transplant, a considerable proportion of *Meis1*-infected cells were found proximal to the transplant, extending up to the S-Z boundary (Fig. 4F; 9 out of 12 cases). In similar transplants using a control *RCAS-GFP* virus, infected cells remained around the wrist level (Fig. 4E; 17 out of 17 cases).

To establish the anatomical contribution of zeugopod RA-treated cells, skeletal morphology was examined at later stages. Only local alterations in limb patterning were observed, and limb development was otherwise completed normally. When beads were incorporated around the S-Z boundary, alterations consisted of segregation of the cartilage surrounding the bead from zeugopodal bones, leading to ectopic joints and reduced radial or ulnar length (Fig. 3C,E,G). In 72% of the cases, the cartilage surrounding the bead fused to the humerus (Fig. 3C,G), generating an extended stylopodal bone that, in some specimens, diverted from the endogenous limb axis, generating an outgrowth (Fig. 3G). Along the zeugopod, RA thus appears to alter the affinity of limb cells, promoting their recruitment to the stylopod. In contrast, beads inserted at stage 25, or later, incorporated into the autopod, provoking its reduction and loss of autopodal structures (not shown). In the autopod, RA thus appears to induce predominantly deletion of distal cells.

In a second series of experiments, RAA beads were used to explore the consequences of inhibiting RA signaling at different levels of PD limb development. RAA beads incorporated proximal to the S-Z boundary provoked reduction or loss of proximal skeletal elements (Fig. 3B,D), while RAA beads that were incorporated distal to the Z-A boundary yielded no alterations in patterning or growth. Unlike RA beads, RAA beads were always found at a similar axial level to control beads and cells around it were not relocated as determined by lineage tracing (Fig. 3D,F,H and not shown). We conclude that the limb can be subdivided into two regions, one proximal to the S-Z boundary, which requires RA and is insensitive to its excess, and another distal to this boundary, which does not require RA and is sensitive to its addition.

RA reprograms limb cells to express a proximalized Hox gene combination

These results suggest that differential RA signaling is important in determining cell identity along the developing PD limb axis. To test this hypothesis, we analyzed expression of Hox genes specific for the main PD limb regions, after experimentally altering RA signaling. *Hoxa11* was used as a marker for the zeugopod and *Hoxa13* as an autopod marker. We first studied the consequences of inserting RA beads subapically at different stages to achieve bead incorporation at different PD axial levels. We found that, when beads incorporated around the presumptive S-Z boundary, *Hoxa11* expression was repressed around the bead, such that its proximal limit of expression was distally displaced (Fig. 5C,E). In contrast, when RA beads were incorporated around

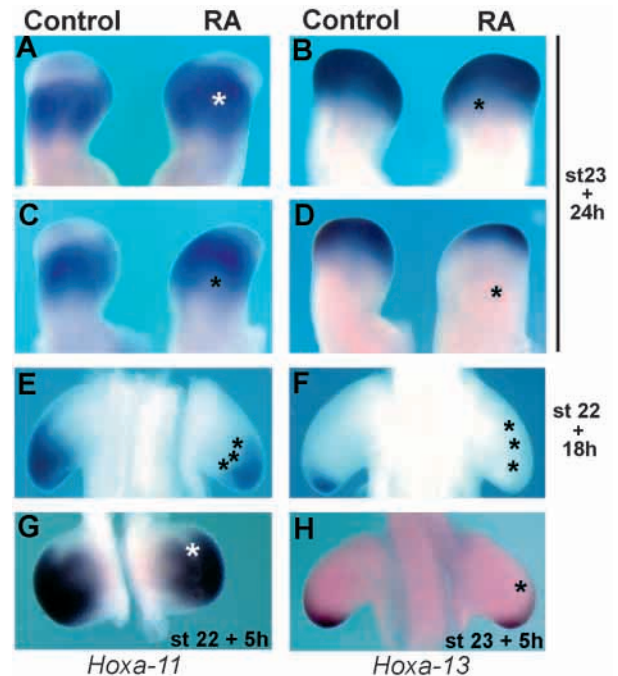


Fig. 5. Retinoic acid (RA) reprograms limb cells to express a proximalized Hox gene combination. Whole-mount in situ hybridizations with *Hoxa11* (A,C,E,G) and *Hoxa13* (B,D,F,H) riboprobes on control limbs (on the left) and RA-treated limbs (on the right). Asterisks indicate bead location. Embryonic stages and time of incubation are indicated on the right or in the pictures.

the presumptive Z-A boundary, *Hoxa11* was activated around the bead, such that its distal limit of expression moved distally (Fig. 5A), and *Hoxa13* expression was repressed, so that its limit of expression also moved distally (Fig. 5B,D), or was even completely repressed (Fig. 5F). We conclude that RA acts by inducing a progressive proximalization of limb cells, forcing them to adopt a phenotype immediately proximal to their position. In contrast to the early activation observed for Meis genes, 5 hours after RA bead implantation no changes in *Hoxa11* expression and only a minimal changes in *Hoxa13* expression were observed (Fig. 5G,H). These results suggest that the changes in Hox gene expression are not the result of direct activation or repression of a particular Hox gene by RA, but rather that a change in PD identity has been induced.

AER signals restrict Meis expression to proximal domains through inhibition of RA signaling

Since Meis expression initially spans the whole lateral plate mesoderm, it follows that all mesenchymal limb cells derive from cells that originally expressed Meis, and some mechanism must restrict Meis expression to proximal limb cells during early limb growth. To determine whether endogenous AER signals are involved in such a mechanism, we removed the AER at stage 20, when the Meis-negative domain has recently appeared. We observed that, by 4 hours after AER removal, Meis expression domains expanded distally, although never reaching the most distal edge of the bud (Fig. 6A,B). This distal extension was completely abolished by inclusion of a distal RAA bead immediately after

AER removal and thus appears dependent on distal extension of the RA effective signaling range (Fig. 6E,F).

Since FGF8 is normally expressed from the AER and can mimic all its properties (Crossley et al., 1996; Vogel et al., 1996), we tested whether it can mediate the AER effects on *Meis* expression. Insertion of an FGF8 bead rapidly repressed *Meis1* in the proximal limb, with kinetics similar to that observed after AER removal or RA signaling inhibition (Fig. 6I,M). FGF8 also repressed *Meis2* expression (Fig. 6N), but repression was slower than in the case of *Meis1*, being barely detectable 4 hours after bead implantation (Fig. 6J). Expression of both genes remained FGF8-sensitive, at least between HH stages 19 to 24. The repression observed was potentiated by the simultaneous insertion of FGF8 and RAA beads (Fig. 6O,P). On the contrary, FGF8 cannot counteract ectopic *Meis* activation by RA in distal limb mesenchyme, suggesting that *Meis* expression status is determined by a balance between the two signals (Fig. 6Q,R). To test for the endogenous FGF signaling requirement in restricting *Meis* expression, we used an inhibitor of FGF signaling (FGFI) that blocks activation of FGF receptor tyrosine kinase activity (Mohammadi et al., 1997). Insertion of FGFI beads subapically produced a very rapid distal expansion of *Meis1* expression domain (Fig. 6C,D). In contrast, *Meis2* expression is only slightly extended in some specimens, suggesting that, in this case, distal factors other than FGFs can restrict *Meis2* expression domain. This observation is in agreement with the lower sensitivity found for *Meis2* repression by added exogenous FGF (Capdevila et al., 1999; Fig. 6J).

As previous studies showed that BMP2 represses *Meis2* expression in the proximal limb (Capdevila et al., 1999), we tested whether *Meis1* was also subject to such regulation. We used BMP2 low-concentration beads to avoid the death-inducing effect of this factor at high concentrations in proximal limb cells. Under these conditions, we found that BMP2 beads induced a rapid repression of *Meis1* expression (Fig. 6G). It was thus possible that repression of BMP signaling may contribute to the observed extension of *Meis* expression after FGFI treatment. We then examined *Bmp2*, *Bmp4* and *Bmp7* expression after FGFI bead insertion. We found that *Bmp2*, *Bmp4* and *Bmp7* expression domains in the posterior limb bud were inhibited, as expected from the demonstrated dependence of BMP on FGF

signalling in this region (Laufer et al., 1994). In contrast, no repression was observed for the *Bmp7* anterior expression domain, and activation was observed for the *Bmp4* anterior expression domain (Fig. 6H,K,L). Since *Meis1* domain extension following FGFI treatment takes place equally in anterior and posterior limb regions, the results suggest that FGF signaling alone can account for *Meis* expression domain restriction, but that other distal signals cooperate in this role during limb development, especially in the case of *Meis2*.

FGFs restrict *Meis* expression by inhibiting RA synthesis and signaling

Our results suggested that FGF signaling inhibits *Meis* expression by blocking RA signaling. To test whether the FGF effect is specific for *Meis* activation by RA, or results from a more general inhibition of the RA pathway by FGF, we studied its effect on *Rarβ*, a direct RA pathway target in limb (Mendelsohn et al., 1992b; Rossant et al., 1991). FGF beads inhibited *Rarβ* expression partially (not shown), although the inhibition was occasionally complete (Fig. 7B) and, conversely, FGFI beads induced the rapid distal expansion of the *Rarβ* expression domain (Fig. 7C). As expected from a RA signaling target, *Rarβ* messenger expression was also rapidly and efficiently inhibited by RAA beads (Fig. 7A). As for *Meis1* expression, RAA and FGF8 acted synergistically to inhibit *Rarβ* in the flank (Fig. 7D). These data suggested that FGF inhibition of *Meis* expression is related to a more general counteraction of the RA signaling pathway.

Expression of the main RA synthesizing enzyme RALDH2 initially spans the whole lateral plate mesoderm but, following limb initiation, it is restricted to the most proximal limb regions

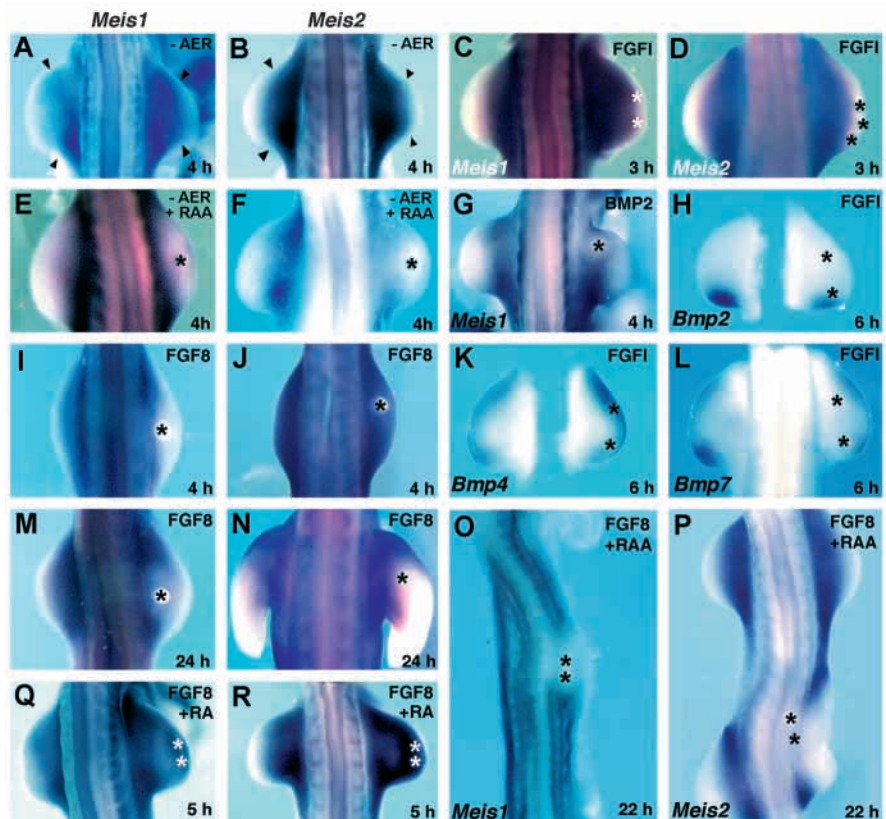


Fig. 6. AER signals restrict *Meis* expression to its proximal domain. *cMeis1* and *cMeis2* whole-mount in situ hybridizations. All embryos were operated at stages 20–22 except those in N (stage 24) and in O (stage 15). Treatment and time of incubation is indicated for each case within the panel. –AER, apical ectodermal ridge removal; FGFI, Fibroblast-growth-factor inhibitor bead; FGF8, Fibroblast-growth-factor 8 bead; RA, retinoic acid bead. BMP2, Bone Morphogenetic Protein 2 bead. Arrowheads in A and B indicate the distal limit of *Meis1/2* expression. Asterisks indicate bead location.

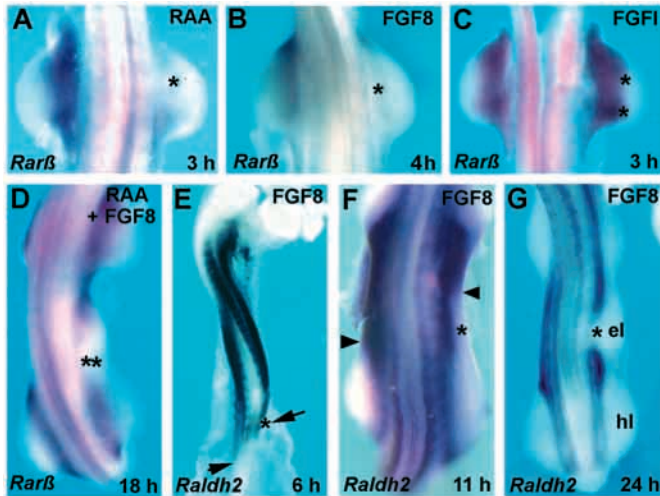


Fig. 7. FGF8 antagonizes RA signaling and synthesis during limb development. (A) RAA represses retinoic acid receptor β (*Rar\beta*) in the proximal limb of a stage 22 limb bud. (B) FGF8 inhibits the expression of *Rar\beta* in the proximal limb bud of a stage 21 embryo. (C) FGFI beads lead to a distal extension of the *Rar\beta* domain at stage 22. (D) Application of FGF8 in combination with retinoic acid antagonist (RAA) potentiates the inhibition of *Rar\beta* in the LPM of a stage 17 embryo. FGF8 inhibits *retinaldehyde dehydrogenase 2* (*Raldh2*) expression (E-G) in lateral plate mesoderm during stages 17-19. Type of bead, time of incubation and riboprobe used are shown in each panel. Asterisks indicate bead location. Arrows in E mark the posterior limit of *Raldh2* expression. Arrowheads in F mark the position where the *Raldh2* expression domain retracts from the distal LPM. hl, hindlimb; el, ectopic limb.

(Swindell et al., 1999) (Fig. 7E-G control left side). This expression profile suggests that signals involved in limb induction may inhibit *Raldh2*, preventing high RA levels in the next limb regions to be generated. Since FGFs are responsible for limb induction, we explored their possible role in *Raldh2* inhibition. We found that FGF8 beads efficiently inhibited *Raldh2* expression when applied at flank level in the early lateral plate mesoderm (Fig. 7E), and subsequently, ectopically extended the *Raldh2*-negative area to the interlimb region (Fig. 7F) and/or generated ectopic repression of *Raldh2* along with the induction of an ectopic limb bud (Fig. 7G). To be effective, *Raldh2* inhibition by FGF8 required at least 6 hours, suggesting that the pathway is not as direct as for Meis and *Rar\beta* repression. In fact, this role of FGF is probably unrelated to the inhibition of the RA pathway, since *Raldh2* is not regulated by RA signaling (Swindell et al., 1999).

These data showed that FGFs produced in the AER are responsible for inhibiting RA function in the distal limb by two different mechanisms, the direct counteraction of RA signaling pathway and the repression of the gene encoding the main RA-synthesizing enzyme.

DISCUSSION

RA is an upstream regulator of Meis during PD development of the vertebrate limb

Exogenously added RA has been shown to cause alterations of

limb patterning in several animals. Local RA application at the anterior limb border induces an additional zone of polarizing activity (ZPA), provoking mirror duplications of the AP limb axis and activation of ZPA-induced Hox genes in the chick (Izpisua-Belmonte et al., 1991; Summerbell, 1983; Tickle et al., 1982). Similar activities were found in the AP axis of regenerating urodele limbs, in which RA also acts as a posteriorizing signal (Kim and Stocum, 1986). Generalized excess of RA produces ectopic or extra limbs in mouse embryos (Niederreither et al., 1996; Rutledge et al., 1994) and frog tail regeneration (Maden, 1993; Mohanty-Hejmadi et al., 1992). These findings point to an early role for RA signaling in limb and/or ZPA induction. In agreement with this proposal, treatment of early limb embryos with RA antagonists (Helms et al., 1996), RA synthesis inhibition (Stratford et al., 1997) or inactivation of the gene encoding the RA-synthesizing enzyme RALDH2 (Niederreither et al., 1999) lead to limb agenesis or truncation. In addition, moderate RA levels in the posterior limb may be required continuously during later development to maintain ZPA activity (Stratford et al., 1997). After limb-bud growth becomes evident, the strong RA signaling area, as defined by target gene and reporter transgene expression, and the RA synthesis area, as defined by *Raldh2* expression, become restricted to proximal limb domains (Berggren et al., 1999; Mendelsohn et al., 1992a,b; Rossant et al., 1991; Swindell et al., 1999).

A principal role for the proximal restriction of strong RA synthesis and signaling in the chick limb, appears to be the maintenance of differential Meis gene expression. This function is essential for the proper specification and patterning of the PD limb axis, and is revealed by the RA effect on PD cell affinity, anatomic contribution of RA-treated cells and reprogramming of the limb PD Hox code by RA. Previous data exploring the role of RA in PD chick limb development, although limited, agree with our findings; RA-treated transplanted PZ cells generate more proximal structures than do control transplants, and late (distal) RA-treated PZ explants do not sort out from early (proximal) PZ explants, as do control explants (Ide et al., 1994; Tamura et al., 1997).

This role of RA in PD specification in the chick limb fits classical experiments in the regeneration of urodele limbs. In the axolotl, limbs amputated at the wrist level regenerate completely from the shoulder region if exposed to RA during regeneration (Maden, 1982). Although RA does not induce obvious PD duplications in the developing chick limb, several experiments in different urodele species fit the findings in the avian model. For example, transplanted blastemas sort out to match the PD levels of host and donor tissues (Crawford and Stocum, 1988) and, in this assay, RA can reprogram distal blastemas to behave as proximal tissue (Crawford and Stocum, 1988). Also in urodele limbs, single distal cells in which retinoic acid receptor γ (RAR γ) was experimentally activated, migrate proximally and contribute to proximal limb compartments (Pecorino et al., 1996). In urodele limb regeneration, RA also induces changes in Hox gene expression that parallel our observations in the chick limb (Gardiner et al., 1995; Simon and Tabin, 1993). If RA can induce similar molecular and cellular changes in developing chick limbs and regenerating urodele limbs, why does it induce complete PD duplications only in the latter model? A possible explanation is that, during normal limb development, reprogrammed cells have the chance to migrate and integrate into proximal

compartments whereas, during regeneration, they are "trapped" in a distal region and thus forced to generate a second proximal compartment. This hypothesis would provide a unified theory of RA function in PD development of vertebrate limbs and predicts a role for Meis genes downstream of RA during urodele limb regeneration.

In the chick limb, RA addition does not impose a determined proximal identity, but rather promotes graded proximalization of limb cells, as for *Meis1* overexpression (Mercader et al., 1999). This raises the question as to whether RA may constitute a morphogen gradient in PD specification, such that different RA concentrations would specify different axial levels. Its ability to induce graded changes in PD specification appear to support this, although this may reflect the intrinsic property of PZ cells to be progressively distalized rather than a morphogen mode of action. Considering that PD identities are acquired by PZ cells in a temporal sequence, RA/Meis activity may act by delaying the normal transition of PZ cells through the different PD identities. In fact, our results using the RAA suggest that, in PD specification, RA is required only in regions proximal to the S-Z boundary and that exogenous RA addition has no effect on patterning within this region. Conversely, distal to the S-Z boundary, RAA does not affect PD limb development, but RA drastically affects patterning and specification. Rather than acting as a morphogen gradient, RA signaling may thus function as a binary switch, subdividing the limb into a proximal, strong RA-signaling area, and a distal, low RA-signaling area. The limit between the two areas, as defined by the threshold for Meis activation by RA, is located at the S-Z boundary. During generation of PD limb identities, repression of RA/Meis activity appears essential to allow identity transition at the S-Z boundary and further limb distalization. It is therefore surprising that both RA and Meis can reprogram PD identity of cells around the Z-A boundary, which is outside their endogenous functional range. This striking result suggests that a similar molecular mechanism may underlie the different identity transitions that take place throughout the PD limb axis generation, such that these transitions remain sensitive to RA/Meis, although endogenously RA only regulates the S-Z transition.

A model integrating signals for limb growth and PD specification

The progress zone model currently explains how limb cells acquire their PD identity in the PZ and become increasingly distalized with time (Summerbell et al., 1973). The first requirement for distalization would be sufficient cell divisions in the PZ. FGFs, as factors essential for PZ cell proliferation, are required for limb cell distalization, and different FGFs can induce the development of a complete limb from embryo flanks triggering the whole limb developmental program, including its distalization (Cohn et al., 1995; Fallon et al., 1994; Niswander et al., 1993). A specific molecular mechanism by which FGFs could regulate limb distalization has not, however, been demonstrated until now. We suggest that FGFs promote limb distalization by counteracting the RA pathway, which is essential to maintain the proximalizing Meis activity in the limb. This FGF activity is achieved by at least two different effects on the RA pathway: inhibition of RA synthesis by repressing *Raldh2*, and parallel direct inhibition of RA signaling, resulting in inactivation of Meis

and other RA targets. As long as AER function is not affected, neither Meis activation, nor RA, inhibit limb growth during the PZ-dependent phase. The role of FGF in repressing RA/Meis pathway therefore does not appear to be related to the promotion of cell proliferation, but rather to a specific function in promoting distalization. In contrast, strong Meis activation in the AER or high RA doses applied directly beneath it, destroy the AER and lead to limb truncations (Tickle et al., 1985, 1989). The AER thus appears to be a structure especially sensitive to RA/Meis activity. The strong expression of the RA-degrading enzyme Cyp26 in the cells delimiting the AER (Swindell et al., 1999) may preserve it from RA/Meis pathway activation during early stages of limb development. Whereas FGF signaling might be the principal and primary signal involved in Meis restriction, other diffusible molecules such as BMP and Wnt, which can also inhibit Meis expression (Capdevila et al., 1999), are likely to cooperate in this role.

We thus propose a model that integrates the roles of FGF in stimulating proliferation of limb cells and in promoting their distalization through inhibition of the strong RA signaling/Meis active domain. The model stresses the relevance of the temporal aspects of PD limb development, to reflect the changing character of the signals and cell domains that take place during limb development (Fig. 8).

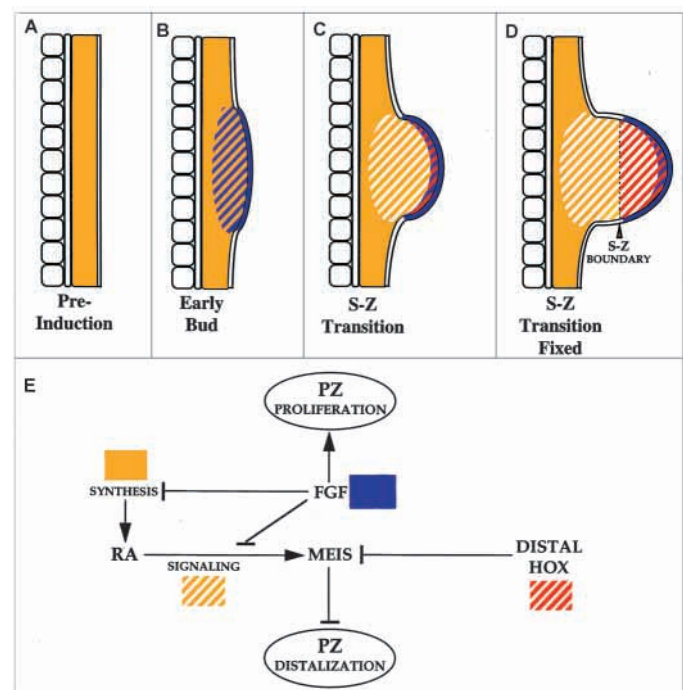


Fig. 8. A new model integrating signals for limb growth and PD specification. (A-D) Different stages of chick limb development. Colored areas mark: RA strong synthesis and signaling area (solid orange), RA signaling domain (hatched orange), fibroblast-growth-factor 8 (FGF8) synthesis and signaling area (blue), region of coexistence of RA and FGF effective signaling ranges (orange bars over blue), and distal Hox gene expression domain (hatched red). Arrowhead in D indicates the stylopod-zeugopod boundary (S-Z boundary). (E) A schematic representation of the interactions taking place between RA and FGF signaling pathways during limb development. Arrows indicate activation and bars indicate repression.

Before limb induction, the whole lateral plate mesoderm represents a region of robust RA synthesis and signaling, strongly expressing Meis genes and other RA targets (Fig. 8A). At limb induction, FGF activity stimulates cell proliferation producing the early limb bud, and also represses RA synthesis from the prospective limb area (Fig. 8B). The early bud retains high enough RA levels to maintain Meis gene activity despite FGF signaling. In agreement with this view, and in contrast to results observed later in the limb bud, FGF8 application to the flank does not repress Meis expression in the most proximal part of the induced limb where RA level is higher (M. A. R., unpublished). The stylopod and more proximal regions, which require both FGF stimulation and RA/Meis activity, are generated during this first phase (Fig. 8B). Early RA functions required for limb and ZPA induction occur up to this stage. As the bud grows, distal cells proliferate and become increasingly distant from the source of RA synthesis, so that their RA concentration is low enough for FGF8 to prevent Meis expression. PZ cells then progress from a stylopodal to a zeugopodal identity, switching off Meis genes and activating zeugopodal genes (Fig. 8C). When, due to the normal elongation of the bud, the FGF8 source later becomes too distant to restrict Meis expression limits, they may be defined by the activity of Hox gene paralogs 10 to 13, which are extensively expressed in zeugopod and autopod and have been shown to repress Meis expression (Capdevila et al., 1999; Fig. 8D). Later in development, an additional principal PD transition takes place between zeugopod and autopod; this transition is independent of, but sensitive to, RA signaling. The regulatory circuit proposed in this model is represented in Fig. 8E.

The antagonism between FGF and RA signaling pathways proposed in this model may not be restricted to limb patterning. This relationship has been suggested to operate during development of several structures, such as hindbrain (Irving and Mason, 2000) or left-right axis specification (Schneider et al., 1999). Further efforts will be required to establish the precise mechanisms by which FGF and RA pathways interplay and the molecular basis of the dual role of FGF in proliferation and distalization of limb cells.

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